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RESEARCH ARTICLE

STUDIES ON THE BACTERIOLYTIC ACTIVITY OF SERUM OF MUD CRAB, *SCYLLA SERRATA* AGAINST THREE DIFFERENT STRAINS OF BACTERIA

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ABSTRACT

Invertebrates are dependent on cellular and humoral immune defences against microbial infection. *Scylla serrata* is an important commercial species of crab, but the fundamental knowledge on its immune defense related to the immune-associated reactions is still lacking. This study was undertaken to determine the antibacterial activities of serum from the mud crab, *Scylla serrata* against three different bacterial strains. Bacterial cultures were treated with different volumes serum from *Scylla serrata* and the growth was monitored by optical density at 450 nm. In addition, the serum was treated with protease to determine the mechanism of antibacterial activities. Treatment of bacterial cultures with serum from mud crab, *Scylla serrata* resulted in a volume-dependent decrease in bacterial growth. Cultures of *M. lysodeikticus,* exhibited strong growth inhibition by serum of *Scylla serrata*, while cultures of V*ibrio furnissi* and *Vibrio damsel* were nearly completely obliterated for 24 h by only 10% (v/v) serum. The antibacterial activity of *Scylla serrata* serum occurred very rapidly, as 18% of *M. lysodeikticus* growth was inhibited by a five min exposure to serum. Furthermore the bacteriolytic activity detected in the presence of phosphate buffer was significantly higher than that observed with, thereby indicating the suitability of phosphate buffer for assay of bacteriolytic activity.

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INTRODUCTION

Immunity to infection is mediated by two systems, namely the acquired or adaptive immune system and the innate or natural immune system. The acquired immune system evolved about 400 million years ago and is found only in vertebrates (Munro *et al*., 1999). The responses are mediated by different agents: macrophages and other phagocytic cells, B and T lymphocytes, antibodies and multitude of other participating proteins. The innate immune system is an ancient form of immunity and offers the main resistance to microbial pathogens within the first minutes, hours or few days of an infection. Invertebrate animals, which lack an adaptive immune system, have developed various defense systems that make-up their so-called “innate immunity” and response to common antigens on the surface of potential pathogen (Khoo *et al*., 1999). Invertebrate defense system is dependent on an innate immune system by a complex of cellular and humoral factors. Invertebrate immunity is efficient in responding to invading foreign materials, utilizing interactive cellular and humoral components (Soderhall and Cerenius, 1996). The primary effector components of the cellular immune system of invertebrates are amoebocytes, coelomocytes or hemocytes (Anbuchezhian *et al*., 2009).

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The major serum factors such as phenoloxidase system, agglutinins, and antimicrobial proteins collectively constitute the humoral immune system in higher invertebrates such as arthropods and molluscs. It is pertinent to note that these humoral components appear to interact with haemocytes and potentiate haemocyte-mediated cellular immune responses against foreign invaders (Chisholm *et al*., 1995). The sera of arthropods are known to contain proteins with antimicrobial properties (Iwanaga *et al*., 2005). The activity of these proteins appear to be directed against a variety of microorganism including Gram-positive and –negative bacteria which is ultimately expressed by bacterial or bacteriolytic effects.

Crustaceans compose a large, ancient and diverse animal group that includes many well-known, commercially exploited members, such as shrimp, crab, crayfish and lobster (Soderhall *et al*., 1996). In normal conditions, crustaceans maintain a healthy state and keep infections under control. Externally, they are covered by a hard, rigid exoskeleton that functions as an efficient physico-chemical barrier against mechanical injury and microbe invasion (Smith *et al.,* 1992). Their gastrointestinal tract, another important route for pathogen invasion, is also protected almost entirely by chitinous membranes. This cuticular coat, in combination with an acid environment rich in digestive enzymes, is able to inactivate and degrade most viruses and bacteria (Battison *et al.,* 2008). However, once the cuticle barriers are disrupted, pathogenic

and/or opportunist microorganisms can penetrate into the haemocoel and thus activate the internal immune defenses of the animal. Both marine and freshwater crustaceans live in an environment often rich in different parasites and pathogens. Therefore, crustaceans must be able to mount an efficient defense against invading pathogenic organisms (Relf *et al*., 1999). In crustaceans, the defense system against microbes rests largely on cellular activities performed by haemocytes, such as cell adhesion, phagocytosis,encapsulation, nodule formation, and melanisation (Majumder *et al*., 1997). In most of the crustacean species studied, the antimicrobial activity has been located in the haemolymph and/or in the haemocytes (Jayasankar *et al*., 1999). However, potent antimicrobial activity has also been detected in other organs/tissues. *Scylla serrata* is an important commercial species of crab, but the fundamental knowledge on its immune defense related to the immune-associated reactions is still lacking. Hence an attempt has been made to investigate the antibacterial activities of serum from the mud crab, *Scylla serrata* against three different bacterial strains and to study the effect of pronase on bacteriolytic activity of unfractionated and fractionated (clarified serum and hemocyanin) serum of *Scylla serrata.*

MATERIALS AND METHODS

Experimental animals and laboratory maintenance

The marine crab *Scylla serrata* weighing 150 to 200 g was obtained from Muttukadu estuary, Chennai. In the laboratory, these crabs were maintained in plastic tanks (90 x 45 x 60 cm) containing aerated natural seawater and the medium was changed every day. The crabs were fed with *Donax spp.* during the period of acclimation (24 h) and only male crabs were used.

Preparation of serum

Haemolymph was collected from the legs of the animal with a fine disposable syringe. To avoid haemocyte degranulation and coagulation, the haemolymph was collected in the presence of Sodium Citrate Buffer, pH 4.6 (2:1 v/v). Equal volume of physiological saline (0.85% NaCl, w/v) was added to it. This was followed by centrifugation at 2000rpm for 15min at 4^oC to remove haemocytes from the haemolymph. Supernatant was collected by aspirating, stored at 4^oC and tested within 16 hours.

Preparation of Microbial Suspension

The pathogenic strains used for the experiment were *Micrococcus lysodeikticus,* V*ibrio furnissi and Vibrio damsela*. 4mg of lyophilized *Micrococcus lysodeikticus* was suspended in 10 ml of sodium phosphate buffer. 4mg of lyophilized V*ibrio furnissi* and 3.3 mg of lyophilized *Vibrio damsela* was suspended separately in 10 ml of phosphate buffer. All bacterial suspensions weres prepared 30 min before use.

Assay of bacteriolytic activity

Bacteriolytic activity of the supernatant obtained from each sample was assayed using bacterial suspension. 200 µl of supernatant from each reaction mixture was added to 1.8 ml of bacterial suspension. A separate tube maintained with 1.8 ml of bacterial suspension and 200 µl of TBS-II served as control. All the tubes were sealed, mixed well and incubated up to 60 min at 26 °C. The optical density was measured at 450 nm against appropriate blank in the spectrophotometer.

RESULTS

Effects of buffer systems on bacteriolytic activity

Bacteria (*M. lysodeikticus*) were suspended in two different buffers namely tris-buffered saline (TBS) and phosphate buffer and the bacteriolytic activity of lysosome (standard) was assayed against bacteria suspended in two different buffer systems (Fig - 1) The bacteriolytic activity detected in phosphate buffer was 307±19 units which is significantly higher than detected in the presence of TBS (p<0.02). Test for bacteriolytic activity of pronase Bacteriolytic activity of pronase was tested against *M. lysodeikticus* (Table). The mean optical density of bacterial suspension was 0.695 at 450 nm. The bacterial suspension concurrently incubated with pronase for 60 min had an optical density of 0.688.

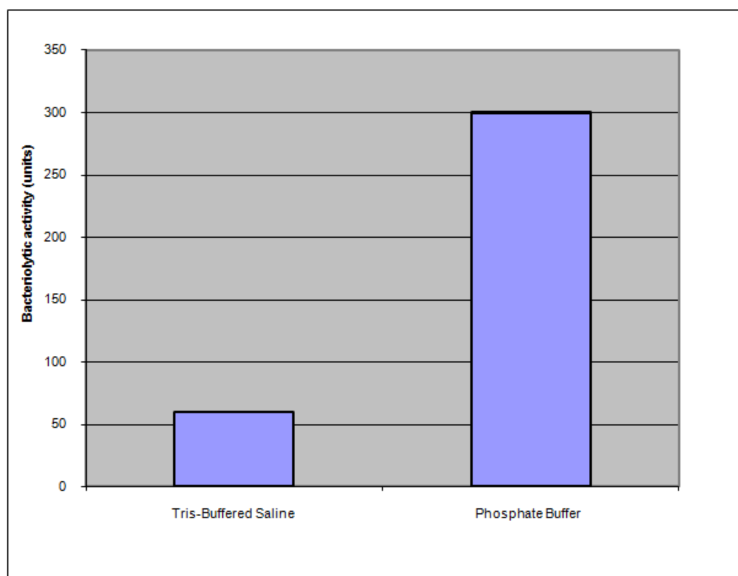

Fig. 1. Effect of buffer system on the bacteriolytic activity of lysozyme (standard : 1.2 g) against *M. lysodeikticus* after 20 min incubation at 26 °C

Assay of bacteriolytic activity at different intervals

The level of bacteriolytic activity in the native serum of *S. serrata* assayed upon incubation with bacterial suspension up to 120 min. the level of serum bacteriolytic activity was 33 units at 30 min, which increased to 78 units at 90 min then declined to 55 units at 120 min. However, rate of the reaction leading to bacteriolytic activity (units/min) was found to be the highest at 60 min.

Pronase – induced bacteriolytic activity

Treatment of unfractionated serum with pronase enhanced the bacteriolytic activity by 152 units against Gram-positive bacteria, *M. lysodeikticus* (Table - 1). A similar treatment of clarified serum and hemocyanin also induced the bacteriolytic activity. However, the enhancement of bacteriolytic activity was much pronounced with clarified serum than in hemocyanin (145 and 46 units, respectively). Both unfractionated serum and clarified serum after pronase treatment exhibited bacteriolytic activity against two types of Gram-negative bacteria, namely, V*ibrio furnissi* and *Vibrio damsela*. These activities are significantly lower ($p<0.01$) as compared to bacteriolytic activity observed against Gram-positive bacteria. Effect of PTU on pronase induced bacteriolytic activity In presence of PTU, pronase induced bacteriolytic activity of unfractionated and clarified serum samples to a level of 212 units and 175 units, respectively. However, these changes are not statistically significant ($p>0.05$) as compared to the effect of pronase observed in the absence of PTU.

buffer was significantly higher than that observed with TBS, thereby indicating the suitability of phosphate buffer for assay of bacteriolytic activity.

Crabs are the very good resource of antimicrobial proteins with wide range of antimicrobial properties which is highly supported in the hemolymph study of *C.*lucifera (Rameshkumar *et al*., 2009). Pronase treated serum exhibited moderate levels of induced bacteriolytic activity against two species of gram-negative bacteria, namely *Vibrio furnissi* and *V. damsela* both of which are known to frequently infect marine crustaceans (Chattopadhyay *et al*., 1996). Upon treatment with pronase, induction of bacteriolytic activity was much prounounced with clarified serum than with hemocyanin, indicating that pronase primarily interacts with some molecules present in clarified serum leading to generation of bacteriolytic activity. Noga,1996 reported the links between the clotting cascade and hemocyanin- based phenoloxidase systems in vitro is not clear, these results suggest that hemocyanin exists abundantly in hemolymph plasma and that it may participate in the innate immune system of the horseshoe crab.The presence of PTU inhibited pronase induced melanization reaction but not bacteriolytic activity. The findings appear to indicate that phenoloxidase generated during melanization reaction upon pronase treatment of serum is not directly responsible for pronase induced bacteriolytic activity in the serum of *S. serrata*. The outcome of this study, nevertheless, elucidates the presence of protease-sensitive inducible humoral immune systems in the serum of *S. serrata*.

Table 1. Effect of pronase on induction of bacteriolytic reaction in the unfractionated serum, clarified serum and hemocyanin of *Scylla serrta*

Bacteria Used	Type of Bacterial strain	Pronase induced bacteriolytic activity(Units)		
		Unfractionated serum	Clarified serum	Haemocyanin
Micrococcus lysodeikticus	Gram-positive	152 ± 70	145 ± 09	46 ±02
V*ibrio furnissi*	Gram-negative	41** ± 90	64** ± 05	Not tested
Vibrio damsela	Gram-negative	30** ± 11	66NS ± 05	Not tested

Table 2. Test for bacteriolytic activity of pronase (5mg/ml) against *M. lysodeikticus* after 60 min incubation at 26 °C

S.No	O.D. of bacterial suspension	
	Without pronase	With pronase
1	0.682 nm	0.680 nm
2	0.712 nm	0.696 nm
3	0.692 nm	0.688 nm
x	0.695 nm	0.688 nm
SD	0.015 nm	0.008 nm

Table 3. Effect of Phenylthiourea on pronase-induced bacteriolytic activity (units) in unfractionated serum, clarified serum of *Scylla serrata*

S.No	Samples tested	Treatment of samples		
		Sample+Buffer	Sample+Pronase	Sample+PTU+Pronase
1	Unfractionated serum	15 ± 8	240*** ± 32 32	212^{NS} ± 23
2	Clarified serum	25 ± 8	152*** ± 28	175^{NS} ± 07

Values represent mean ±SD from 4 determinations

One unit of bacteriolytic activity is defined as the decrease of 0.001 adsorbancy at 450 nm of bacterial suspension in 60 min upon addition of sample

* Asterisk indicates statistical significance: *** $p<0.002$; NS: not statistically significant; mean difference Student *t*- test

DISCUSSION

In the present study, an attempt was made to elucidate generation of immunologically reactive molecules-antimicrobial proteins in the serum of *S. serrata*. The bacteriolytic activity detected in the presence of phosphate

These reactions can very well serve adaptive immune functions against potential pathogens in the marine crab *Scylla serrata.* The present study revealed that the haemolymph might act as storage of antimicrobial peptides. In conclusion, the present study indicates that the haemolymph of *S. serrata* would be a good source of antimicrobial agents and can be used as cost

effective antibiotics. The present antimicrobial assays conducted will serve as a baseline data for further studies that may confirm the hypothesis that the hemolymph of crabs are indeed potential source of anti microbial compounds with biological potential.

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